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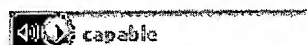
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capable



ca·pa·ble [káypəb'l]

adjective

- doing something well:** good at a particular task or job or at a number of different things
 - a very capable hotel manager*
- able to do particular thing:** possessing the qualities needed to do a particular thing
- liable to:** permitting or susceptible to something
 - an action capable of being misinterpreted*
- LAW legally competent:** the ability or the legal power to do something

[Mid-16th century. Via French from late Latin *capabilis*, from Latin *capere* "to take." Originally "able to take in."]

- ca·pa·ble·ness *noun*

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capable

Main Entry: **ca·pa·ble**

Pronunciation: 'kA-p&-b&l, in rapid speech 'kAp-b&l

Function: *adjective*

Etymology: Middle French or Late Latin; Middle French *capable*, from Late Latin *capabilis*, irregular from Latin *capere* to take -- more at [HEAVE](#)

1 : SUSCEPTIBLE <a remark *capable* of being misunderstood>

2 obsolete : COMPREHENSIVE

3 : having attributes (as physical or mental power) required for performance or accomplishment <is *capable* of intense concentration>

4 : having traits conducive to or features permitting <this woman is *capable* of murder by violence -- Robert Graves>
<an outer coat of light color *capable* of reflecting solar heat -- *Current Biography*>

5 : having legal right to own, enjoy, or perform

6 : having general efficiency and ability

- **ca·pa·ble·ness** /'kA-p&-b&l-n&s/ *noun*

- **ca·pa·bly** /-p&-blE/ *adverb*

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PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:
Barrett R. Harvey *et al.*

Serial No.: 10/620,278

Filed: July 15, 2003

For: COMBINATORIAL PROTEIN LIBRARY
SCREENING BY PERIPLASMIC
EXPRESSION

Group Art Unit: 1645

Examiner: Ford, V.

Atty. Dkt. No.: UTXB:715US

DECLARATION OF BARRETT R. HARVEY UNDER 37 C.F.R. § 1.132

Assistant Commissioner for Patents
Washington, D.C. 20231

I, BARRETT R. HARVEY, HEREBY DECLARE AS FOLLOWS:

1. I am a citizen of the United States, and currently reside at 203 Green St., Souderton, PA 18964.
2. I was employed by The University of Texas from 1998 until 2004, with the title of Research Assistant. During that time I was a graduate student at The University of Texas. My primary duties as a Research Assistant were in conducting scientific research in the area of molecular biology. My supervisor was George Georgiou, Ph.D., who is a co-inventor of the above captioned patent application. I am currently employed by Merck & Co., Inc.

2. I received a Ph.D. in Molecular Biology from The University of Texas in December, 2003. I have been conducting research in the area of microbiology since 1998. I have a Bachelors degree in Microbiology from The University of Texas.

3. I am a co-inventor of the above captioned patent application, have reviewed the amended claims, and am familiar with the technology and steps described in the amended claims.

4. I understand that the Patent and Trademark Office Examiner in charge of assessing the patentability of the referenced patent application has rejected the claims of the application for failing to enable a person of ordinary skill to use fragments of transmembrane proteins and fragments of inner membrane lipoproteins for practicing the method described in claim 1 of this application.

5. I am therefore providing the present Declaration to demonstrate that the method described in claim 1 is enabled for the use of fragments of transmembrane proteins and fragments of inner membrane lipoproteins.

6. The studies carried out in working examples 1-4 and 15 of the above-captioned invention demonstrate that claim 1 is enabled for the use of fragments of transmembrane proteins and fragments of inner membrane lipoproteins as follows:

A. Fragments of inner membrane lipoproteins

Examples 1-4 confirm that the method of claim 1 can be achieved by anchoring a binding polypeptide to essentially any fragment of an inner membrane lipoprotein. Specifically, these examples demonstrate the use of a six amino acid fragment of new lipoprotein A (NlpA) to anchor

single chain variable fragment (scFv) antibodies to the periplasmic face of the inner membrane. As with all lipoproteins, the localization of NlpA to the inner membrane of a prokaryotic cell is determined by the presence of Aspartate in the position of the second amino acid residue (see specification page 17 lines 23-26). Since changing amino acid 2 of any lipoprotein or lipoprotein fragment to an Aspartate will target it to reside in the inner membrane, the specification enables targeting of essentially any lipoprotein or lipoprotein fragment to the inner membrane (see specification page 18 lines 1-4). Therefore, the application fully teaches one of skill in the art to use fragments of lipoproteins to practice the method of claim 1 with only routine experimentation.

B. Fragments of transmembrane proteins

Example 15 confirms that the method of claim 1 can also be achieved by anchoring a binding polypeptide to a fragment of transmembrane proteins with only routine experimentation. Specifically, the example demonstrates the use of a fragment of the phage gene 3 minor coat protein (g3p), referred to as the "short variant" of g3p, to anchor a scFv antibody to the periplasmic face of the inner membrane. During normal phage morphogenesis, g3p becomes positioned across the inner membrane before it is incorporated onto the growing phage. Thus expression of the short variant of g3p without any other phage genes causes the g3p fragment to remain attached to the inner membrane as a transmembrane protein. Example 15 therefore demonstrates that the method of claim 1 can be achieved by anchoring a binding polypeptide to a fragment of essentially any transmembrane protein.

7. Further studies have demonstrated that fragments of the TatC protein, and thus any transmembrane protein fragment, can be used as anchors to achieve the method of claim 1. These studies, which were prepared by Ki Jun Jeong along with my participation and under

the supervision of George Georgiou, a co-inventor in the above captioned patent application, were carried out as follows:

A. Fragments of TatC

A library of fragments of the *E. coli* transmembrane protein TatC was constructed by use of the thio-ITCHY method to generate deletions in the TatC gene. The resulting gene fragments were ligated to a vector containing the 26-10 scFv antibody, which is a digoxin specific binding protein. The resulting library contained fusions spanning the entire TatC gene, which allowed screening of over 2.0×10^4 different fragments as is described below.

B. Screening of fragments

The TatC-scFv library of fusions was expressed in *E. Coli*, labeled with digoxin-BODIPY™, and highly florescent clones were isolated following two rounds of fluorescence activated cell sorting (FACS) using the anchored periplasmic expression technique as described in this patent application. The sorted cells were incubated on agar plates, and 40 random clones were chosen for investigation of their individual florescence intensity. All 40 clones showed up to 10 times higher mean cell fluorescence then negative controls, and sequencing of the 40 clones revealed 28 unique fusion junctions. This demonstrated that at least 28 different fragments of the TatC transmembrane protein could be used as anchors to practice the method of claim 1. The figure attached as **Exhibit A** illustrates the location and identity of the 28 different fusion points between the TatC fragments and the binding protein. This figure demonstrates that in over 95% of the selected clones (38 out of the tested 40 clones), the scFv was fused to sites that were periplasmic or to sites that were near the periplasmic end of a transmembrane helix.

C. Conclusion

The foregoing demonstrates that those with ordinary skill in the art can create and screen a library of fragments from any transmembrane gene, illustrating that the method of claim 1 can be achieved using fragments from any transmembrane protein with only routine experimentation.

8. Based on the working examples and studies discussed above, a person of ordinary skill in the art in possession of the application can practice the method described in claim 1 of the patent application using a broad range of fragments from lipoproteins as well as fragments from transmembrane proteins. Only routine experimentation would be required to use fragments of lipoproteins or fragments of transmembrane proteins to practice the method described in claim 1.

9. I hereby declare that all statements made herein of my knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

9/14/04
Date

Barrett R. Harvey
Barrett R. Harvey

PERIPLASM

CYTOPLASM

Diagram illustrating the primary structure of the outer membrane protein OmpA, showing the sequence of amino acids (represented by letters in circles) and their positions relative to the periplasm and cytoplasm. The structure is divided into two main regions: PERIPLASM (top) and CYTOPLASM (bottom). The sequence is numbered from 1 to 258. Key residues are highlighted with different symbols: open circles for standard amino acids, filled circles for hydrophobic residues, and squares for charged residues. The protein has a complex topology with multiple transmembrane segments and loops.

The diagram illustrates the structure of the outer membrane of *E. coli*, showing the periplasm and cytoplasm. The diagram is divided into two main regions: the PERIPLASM (top) and the CYTOPLASM (bottom). The outer membrane is shown as a phospholipid bilayer with various proteins embedded within it. The proteins are represented by circles containing their amino acid residues. The diagram shows the arrangement of various proteins and lipids, including the outer membrane, periplasm, and cytoplasm. Key components labeled include: PERIPLASM, CYTOPLASM, and various amino acid residues (e.g., P, Q, G, S, L, Q, K, I, V, S, A, P, L, L, H, Y, I, D, F, A, N, F, V, Y, L, C, F, V, L, I, A, V, N, C, L, L, R, K, R, L, E, I, L, H, T, I, P, D, T, Q, E, V, S, M, NH₂, A, T, D, V, F, P, S, A, F, I, P, L, K, I, T, F, M, V, S, L, I, L, S, A, P, V, I, L, Y, Q, V, W, A, P, R, Y, H, R, R, E, V, P, L, V, S, S, S, L, L, V, P, T, M, A, L, F, G, V, M, C, V, A, F, G, V, M, F, V, A, L, V, L, C, W, M, T, I, G, S, P, E, D, L, K, K, R, 190, 250, 258, COOH).

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:
Gang Chen, et al.

Serial No.: 09/699,023

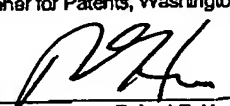
Filed: October 27, 2000

For: ISOLATION OF BINDING PROTEINS
WITH HIGH AFFINITY TO LIGANDS

Group Art Unit: 1632

Examiner: Ford, V.

Atty. Dkt. No.: UTSB:675US

CERTIFICATE OF MAILING 37 C.F.R. §1.8	
I hereby certify that this correspondence is being deposited with the U.S. Postal Service with sufficient postage as First Class Mail in an envelope addressed to: Assistant Commissioner for Patents, Washington, DC 20231, on the date below.	
<u>05/04/04</u> Date	 Robert E. Hanson

DECLARATION OF JONGSIK GAM UNDER 37 C.F.R. § 1.132

Assistant Commissioner for Patents
Washington, D.C. 20231

I, JONGSIK GAM, HEREBY DECLARE AS FOLLOWS:

1. I am a citizen of South Korea, and currently reside at 1014 Stratford Ct., State College, PA.
2. I was employed by The University of Texas from 1997 until 2004, with the title of Research Assistant. During that time I was a graduate student at The University of Texas. My primary duties as a Research Assistant were in conducting scientific research in the area of

25407745_1.DOC

pharmacy. One of my two supervisors was Brent Iverson, Ph.D., who is a co-inventor of the above captioned patent application.

2. I will receive a Ph.D. in Pharmacy from The University of Texas in May, 2004. I have been conducting research in the area of pharmacy since 1997. I have a Bachelors degree and a Masters degree in Pharmacy from the Seoul National University in Seoul, South Korea.

3. I have reviewed the amended claims of the above-captioned patent application and am familiar with the technology and steps described in the claims.

4. I understand that the Patent and Trademark Office Examiner in charge of assessing the patentability of the referenced patent application has rejected the claims of the above patent application for failing to recite a wash step. In particular, it is my understanding that the Examiner asserts that a separate wash step must be added to make the following procedure functional:

1. (Current amended) A method of obtaining a bacterium comprising a nucleic acid sequence encoding a binding protein capable of binding a target ligand comprising the steps of:

- (a) providing a Gram negative bacterium comprising a nucleic acid sequence encoding a candidate binding protein, wherein said binding protein is expressed in soluble form in the periplasm of said bacterium;
- (b) contacting said bacterium with a labeled ligand capable of entering said periplasm; and
- (c) selecting said bacterium based on the presence of said labeled ligand within the periplasm, wherein said ligand and said candidate binding protein are bound in said bacterium.

5. I am providing the present Declaration to submit data demonstrating that a wash step is not required for the successful use of the above procedure.

6. The studies carried out by me demonstrating that a wash step is not necessary for the function of the technique described in the claims can be summarized as follows:

A. Bacteria

E. coli ABLETMC and TGI bacteria were obtained expressing a digoxigenin-specific scFv in soluble form in the periplasm. Periplasmic expression was achieved by linking the scFv to a *pelB* leader sequence. Expression was regulated by the *lac* inducible promoter, the expression of which is induced by the addition of IPTG to growth media.

B. Cell culture

Growth media was supplemented with ampicillin (100 µg/ml) when necessary. Bacteria containing the plasmid encoding the scFv were grown overnight in TB broth containing 2% glucose at 30°C. Cells were subcultured in fresh 2xYT (or LB) media and re-grown at 37°C for 2 hours until an OD600 of 0.5-0.8 was reached. The cells were induced with IPTG (0.2 mM) for an additional 4 hours at 25°C to express the scFv antibodies in the periplasm of the *E. coli* strains.

C. Labeling

50 µl of the induced cell cultures were incubated in 950 µl of 1xPBS at room temperature for 1 hour with BODIPY-conjugated digoxigenin (usually 200 nM = 0.2 µM). The incubation was performed successfully with and without use of permeabilizers (PMBN, EDTA or lactic acids were used when a permeabilizer was included). At this stage, trials were run both with and without a washing step after the incubation. The resulting cell suspension (10 µl without a washing step), was diluted with 1 ml of 1xPBS in a SIP tube for the FACS analysis.

D. Selection

10⁴ events were analyzed on a Becton Dickinson FACSCalibur for both the washed and unwashed trials. Sheath flow was 1xPBS for all of the screens. Cell viability was judged using the propidium

iodide (PI) staining method (1 μ g/ml). The screening demonstrated a high FACS signal relative to negative controls lacking the digoxigenin-specific scFv both with and without the wash step. The results confirmed that in cells expressing the scFv binding protein with specific affinity for the labeled ligand the bound complex was accumulated and becomes detectable above background levels (the control) based on this concentration of signal regardless of the presence of unbound labeled ligand.

7. Based on the foregoing studies, a wash step is not required for the use of the method described in the claims of this patent application to obtain a bacterium comprising a nucleic acid sequence encoding a binding protein capable of binding a target ligand.

8. I hereby declare that all statements made herein of my knowledge are true and that all statements made on information and belief are believed to be true, and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

5/4/04

Date

Jongsik Gam

Jongsik Gam

Isolation of high-affinity ligand-binding proteins by periplasmic expression with cytometric screening (PECS)

Gang Chen^{1†}, Andrew Hayhurst^{1,2†}, Jeffery G. Thomas^{2,3}, Barrett R. Harvey¹, Brent L. Iverson^{1,4*}, and George Georgiou^{1,2,5*}

Periplasmic expression with cytometric screening (PECS) is a powerful and rapid "display-less" technology for isolating ligand-binding proteins from diverse libraries. *Escherichia coli* expressing a library of proteins secreted into the periplasmic space are incubated with a fluorescent conjugate of the target ligand. Under the proper conditions, ligands as large as about 10 kDa can equilibrate within the periplasmic space without compromising the cell's integrity or viability. The bacterial cell envelope effectively serves as a dialysis bag to selectively retain receptor-fluorescent probe complexes but not free ligand. Cells displaying increased fluorescence are then isolated by flow cytometry. We demonstrate that scFv antibodies with both very high and low affinity to digoxigenin can be isolated from libraries screened by PECS using a benchtop flow cytometer. We also show that preexisting libraries constructed for display on filamentous bacteriophage can be screened by PECS without the need for subcloning. In fact, PECS was found to select for proteins that could be missed by conventional phage panning and screening methods.

Protein display technologies collectively represent one of the most powerful tools for protein engineering¹. For display purposes, a protein is fused to the C or N terminus of a polypeptide sequence that targets the resulting chimera onto the surfaces of biological particles such as viruses, bacteria, yeast, or higher cells. Libraries are typically screened for ligand binding by a series of adsorption-desorption cycles, a process also known as "panning"². Panning has been used successfully to screen highly complex libraries made by cloning the mammalian antibody repertoire and displaying it on phage (up to 10¹¹ clones). For somewhat less diverse libraries (up to 10⁶ clones), display on bacteria or yeast coupled with flow cytometry is a powerful tool for the discovery of proteins with exceptionally high ligand-binding affinities and for the isolation of enzyme catalysts³⁻⁵.

Although the importance of display technologies for protein engineering is undisputed, the need to anchor the target polypeptide onto the surface of a biological particle imposes a number of limitations that can significantly reduce the diversity of the library relative to the totality of proteins that can be produced in a soluble form within the cell. First and foremost, protein display requires that the protein of interest be expressed as either a C- or N-terminal fusion, a process that can adversely affect protein function or stability. Second, protein display is subject to biological constraints associated with protein export and presentation, which may compromise the viability of the virus or cell (e.g., refs 6,7). Third, complex proteins consisting of several polypeptide chains or requiring certain post-translational modifications may be intrinsically incompatible with display on phage, bacteria, or yeast. Such proteins include, for example, cofactor-containing bacterial proteins whose biosynthesis depends on the recently discovered twin arginine motif export system⁸. Fourth, display can introduce certain complications for screening such as avidity effects⁹⁻¹¹, in the case of viruses, or nonspecific binding of the fluorescent probe to the cell surface⁴.

Here we describe a "display-less" approach for isolating proteins that bind to small molecules, including peptides and oligonucleotides, based on PECS. Libraries are simply expressed in soluble form in the periplasmic space of *E. coli* by means of a suitable signal sequence and mixed with a fluorescently tagged ligand (Fig. 1). Normally, the outer membrane of Gram-negative bacteria restricts the diffusion of molecules larger than ~650 Da¹². However, we have found that an appropriate combination of growth conditions and bacterial strains permits molecules as large as 10 kDa to equilibrate through the outer membrane while periplasmically expressed proteins are retained within the cell. Specific binding of the fluorescent conjugate by the cognate periplasmic protein results in increased fluorescence, allowing the cells to be isolated from the rest of the library by fluorescence-activated cell sorting (FACS). Thus the cell envelope serves as a dialysis bag that retains protein-ligand complexes.

We have demonstrated the utility of PECS for discovering ligand-binding scFv antibodies. One advantage of PECS is that it can readily be used to screen by flow cytometry any existing library constructed for display on bacteriophage without the need for subcloning. This feature allowed the isolation of unique binding clones that could not be identified by conventional phage panning.

Results and discussion

Fluorescence detection and enrichment of cells expressing scFv antibodies in the periplasm. The 26-10 scFv antibody binds with high affinity to cardiac glycosides such as digoxin and digoxigenin (the *K_d* values of the purified antibody for digoxin and digoxigenin are $0.9 \pm 0.2 \times 10^{-9}$ M and $2.4 \pm 0.4 \times 10^{-9}$ M, respectively¹³). The 26-10 scFv and its variants have been used extensively as a model system to understand the effect of mutations in the complementarity-determining regions (CDRs) and in the framework regions on hapten binding¹³⁻¹⁶. We expressed the 26-10 scFv in the *E. coli* periplasm

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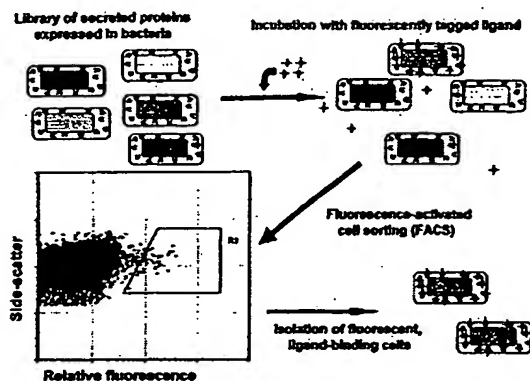


Figure 1. A schematic diagram showing the principle of periplasmic expression with cytometric screening (PECS). Libraries of proteins secreted to the *E. coli* periplasm can be labeled by fluorescent conjugates of ligands by a simple high-salt incubation procedure. The outer membrane serves as a dialysis membrane allowing diffusion of unbound ligand out of the cell and retention of protein-ligand complexes within the cell. The fluorescent cells are then separated by FACS according to user-defined parameters (e.g., region one: R1). Only cells expressing protein species with the desired ligand-binding characteristics are enriched.

using the arabinose promoter and *pelB* leader sequence in plasmid pBAD30*pelB*-Dig. This vector was used to transform the *ara* *E. coli* strain LMG194, and scFv expression was induced with 0.2% (wt/vol) arabinose at 25°C. We observed that upon incubation with 100 nM of digoxigenin-BODIPY, the cells became strongly fluorescent, and the fluorescence signal was retained even after extensive washing to remove nonspecifically bound ligand (data not shown). Staining with the viability stain propidium iodide (PI), which binds specifically to damaged cells by intercalating in normally inaccessible double-stranded nucleic acids, revealed that >90% of the cells were not permeable to the dye. The fraction of viable cells was similar to that in control cultures harvested in late exponential phase, indicating that the ligand-bound *E. coli* cells were structurally intact.

Cells expressing the 26-10 scFv antibody in the periplasm could be enriched from a large excess of *E. coli* transformed with vector alone in a single round of sorting. Specifically, LMG194 (pBAD30*pelB*-

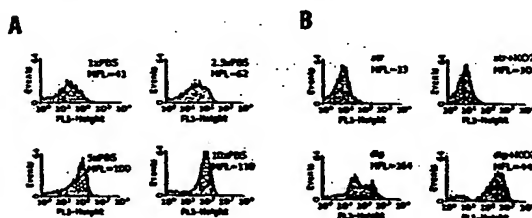


Figure 2. Optimization of the periplasmic expression screening signal. (A) Effect of hyperosmotic shock on efficiency of labeling of the 26-10 scFv expressed from pHEN2.dig26-10 in *E. coli* ABLEC and labeled for 1 h with 100 nM digoxigenin-BODIPY. The PBS concentration employed and the mean fluorescence (MFL) of the populations are shown. Cells expressing unrelated antibodies (to atrazine or thyroglobulin) and labeled under identical conditions gave a MFL of 5-6 (data not shown). (B) Effect of infection with M13K07 on the fluorescence signal detected in ABLEC cells labeled with 100 nM digoxigenin-BODIPY in 5x PBS. Cells were expressing either an anti-atrazine scFv (atr) or the 26-10 scFv (dig) using the powerful P_{lac} promoter and either left uninfected or infected (K07) at a multiplicity of infection of 10 at 0.5 h preinduction with 1 mM IPTG and selection for phage infection with kanamycin (70 µg/ml).

Table 1. Light-chain CDR3 amino acid sequences of mutants*

	Light-chain sequence	Off-rate (s^{-1})
	90-----96	
A4-19	Q T T H V P P	2.7×10^{-4}
60.1 (1)	Q T T H S P A	5.5×10^{-4}
60.2 (2)	Q T T H L P T	2.8×10^{-4}
60.3 (1)	Q T T H T P P	ND
60.4 (1)	Q T T H L P A	ND
90.1 (1)	Q T T H I P T	3.2×10^{-4}
90.2 (1)	Q T T H V P P	2.7×10^{-4}
90.3 (7)	Q T T H V P A	2.2×10^{-4}
90.4 (1)	Q T T H I P A	1.4×10^{-4}
90.5 (3)	Q T T H L P A	ND
90.6 (1)	Q T T H V P C	ND

*Mutants were isolated by 60 min (60.1-60.4) and 90 min (90.1-90.6) off-rate selection with the actual number of identical clones isolated in parentheses. ND, No data.

Dig) cells were mixed with a 10,000-fold excess of *E. coli* containing vector devoid of an scFv insert (pBAD30). After fluorescence labeling and cell sorting, enrichment factors of over 1,000 fold per round were obtained (data not shown). Cells expressing control scFv as opposed to empty vector were also unstained by the labeling procedure (see below and Fig. 2).

Antibody affinity maturation using PECS. An affinity-improved variant of the 26-10 scFv containing three amino acid substitutions in heavy-chain CDR1 (T30P, D31S, and M34Y) was isolated¹⁵. This clone was designated A4-19. Using surface plasmon resonance, we determined the equilibrium dissociation constant (K_D) of the purified, monomeric A4-19 scFv to be 0.3 nM compared to 0.9 nM for the 26-10 scFv parental antibody. Three light-chain CDR3 residues that make contact with (Thr91, Pro96) or are in close proximity to (Val94) the digoxin hapten¹⁷ were randomized using an NNS ($S = G$ or C) strategy¹⁸. A library of 10^6 transformants was obtained in the pBAD*pelB* vector. The library was screened by two rounds of FACS using a low-throughput benchtop flow cytometer (Becton Dickinson FACSsort). In the first round of screening, the labeled cells were washed once with PBS and sorted in recovery mode, which collects all fluorescent events including droplets that contain both a nonfluorescent and fluorescent particle. Operation in recovery mode collects more rare cells at the expense of purity. The collected cells were regrown, labeled, washed, and then incubated with a 50-fold excess (5 µM) of free digoxin for various times. Cells that retained the desired level of fluorescence were isolated by FACS using exclusion mode, which ensures a higher degree of purity by rejecting coincident fluorescent and nonfluorescent events. Five random clones from the cell population isolated following incubation with competitor for 60 min and 13 clones from the 90 min pool were picked at random and sequenced (Table 1). The respective scFv antibody proteins were purified by Ni affinity chromatography followed by gel filtration fast-performance liquid chromatography (FPLC), and the hapten-binding kinetics of the monomeric scFv proteins were determined by surface plasmon resonance. All the scFvs examined displayed association rate constants (k_{on}) indistinguishable from those of the starting A4-19 antibody ($0.9 \pm 0.2 \times 10^6 M^{-1}s^{-1}$). The k_{on} of the clones isolated after 60 min of competition were the same as or faster than that of A4-19. However, two out of three clones isolated after a 90 min competition exhibited slower k_{on} values, with one clone, 90.4, having a twofold slower dissociation rate constant resulting in a K_D of 150 pM. Thus, we could isolate a higher affinity mutant of an antibody that already exhibited a subnanomolar K_D by screening a library of 10^6 clones using only two rounds of PECS.

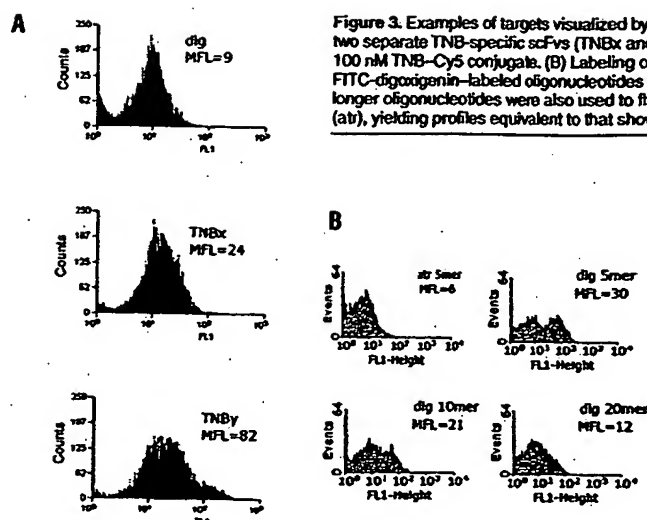


Figure 3. Examples of targets visualized by PECS. (A) Fluorescence distribution of ABLEC cells expressing two separate TNB-specific scFvs (TNBx and TNBy) and the control 26-10 scFv (dig) labeled in 5x PBS with 100 nM TNB-Cy5 conjugate. (B) Labeling of cells expressing the 26-10 scFv (dig) with 100 nM FITC-digoxigenin-labeled oligonucleotides that are 5 (5mer), 10 (10mer), or 20 (20mer) bases long. The longer oligonucleotides were also used to fluorescence-label cells expressing the anti-atrazine control scFv (atr), yielding profiles equivalent to that shown for the 5mer.

Optimization of labeling conditions and application to other ligands. To achieve optimal enrichment ratios during library screening by PECS, it is important to obtain the maximum fluorescence signal possible without compromising cell viability¹⁸. The fluorescent intensity of cells expressing binding proteins in the periplasm and incubated with ligand concentrations above the K_D was found to depend on a number of physiological and genetic parameters. A significant increase in fluorescence with concomitant retention of cell viability was observed when the cells were grown at 25°C. Little soluble scFv is produced at 37°C (e.g. ref. 19), and growth at subphysiological temperatures generally enhances the yield of correctly folded proteins. Importantly, growth at 25°C increases the permeability of the outer membrane, in part because the cold-sensitive secretory pathway alters the membrane composition²⁰.

Various *E. coli* strains expressing the 26-10 scFv exhibited significant differences with respect to labeling with 100 nM digoxigenin-BODIPY. For example, the commonly used library strains TG-1 and HB2151 displayed a mean fluorescence intensity only threefold higher relative to the respective control cells containing vector alone. The highest signal (eightfold higher than the control) was obtained with *E. coli* ABLEC under a variety of induction regimes and growth conditions. In all media (LB, TB, 2x TY), at all temperatures (20°, 25°, and 30°C), with all combinations of temperature shifts upon induc-

tion (37°–25°, 37°–30°C, no shift), and with all levels of IPTG induction (10, 100, 1,000 µM), ABLEC surpassed the other strains tested.

The ionic strength and composition of the buffer used for labeling also had a pronounced effect on the mean fluorescence intensity of the cells (Fig. 2A). Labeling in 5-fold or 10-fold concentrated PBS resulted in a more uniform and highly fluorescent population. Labeling in 5x PBS gave a peak-to-peak separation between control and 26-10 scFv-expressing cells of around 20-fold. The elevated salt concentration and osmotic pressure under these conditions may be disrupting the ordered nature of the lipopolysaccharide layer in the outer membrane, resulting in increased permeability^{21,22}. Whereas 10x PBS gave the highest mean fluorescence intensity, cell viability (as judged by PI staining) tended to be compromised, and so we typically used 5x PBS. The highest fluorescent signals were obtained by (1) driving the expression of scFv antibodies from the more powerful P_{lac} promoter; (2) using ABLEC as the host strain; (3) labeling in 5x PBS buffer; and (4) infecting the cells with the filamentous phage M13K07 (Fig. 2B). Although pIII (a minor coat protein) expressed in the absence of other phage proteins has been shown to increase the permeability of the outer membrane²³, overexpression of pIII is extremely toxic to the host cell, especially when it is fused to scFv (ref. 24). Likewise, although mutants of the large aqueous pore-forming pIV have been shown to improve permeability to ligands above the 650 Da limit²⁵, its overexpression induces the phage shock response²⁶, adversely affecting cell growth. Thus, although phage infection resulted in the highest fluorescent intensities without reducing cell viability, we generally avoided it because of much slower growth of infected versus uninfected cells.

Using optimal conditions, cells expressing scFvs in the periplasm could be fluorescently stained with a variety of ligands including small molecules, oligonucleotides, or peptides with molecular weights as high as 10,000 Da (Fig. 3). For example, Figure 3A shows the fluorescence distribution of cells expressing two different scFv antibodies to trinitrobenzene (TNB) isolated from a naïve library (A. Hayhurst, B.R. Harvey, B.L. Iverson, and G. Georgiou, unpublished results). The cells were stained with the fluorescent conjugate

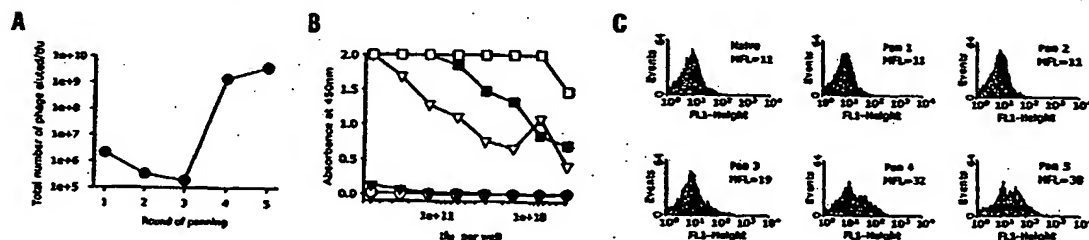


Figure 4. Monitoring the phage panning process using FACS via PECS. (A) Phage eluate titers after each round of panning the Griffin library on digoxin-BSA conjugate. t.u., Transforming units. (B) Polyclonal phage ELISA of purified phage stocks from each round of panning on digoxin-ovalbumin. Points are the average of duplicate wells. The naïve signal is overshadowed by the pan1 signal. Naïve library (filled circles); pan 1 (empty circles); pan 2 (inverted filled triangles); pan 3 (inverted empty triangles); pan 4 (filled squares); pan 5 (empty squares). (C) Periplasmic expression screening of a portion of the naïve Griffin library and rounds 1 to 5 from a panning experiment following infection of ABLEC and labeling in 5x PBS with 100 nM digoxigenin-BODIPY.

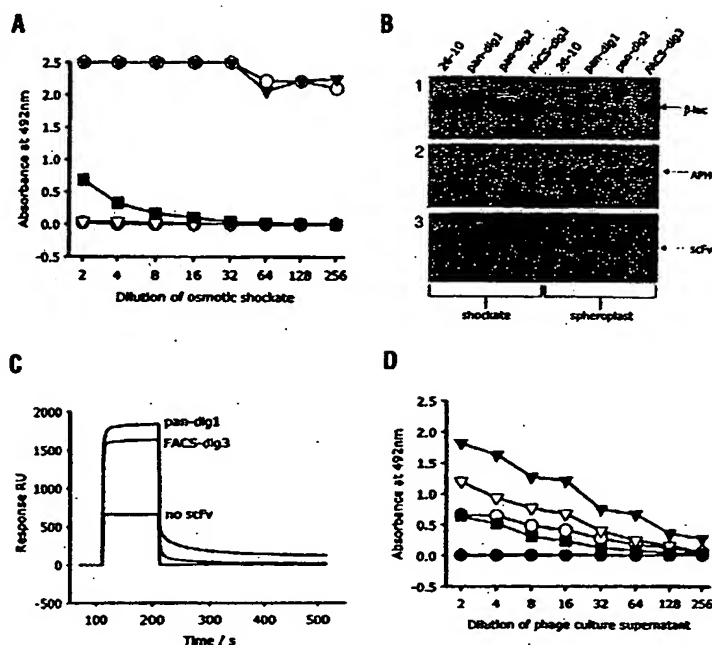


Figure 5. Examining clones isolated by phage display and PECS. (A) Soluble scFv ELISA signals from osmotic shock fluid of ABLEC containing pHEN2 with negative control anti-atrazine scFv (filled circles); positive control 26-10 (empty circles); pan-dig1 (inverted filled triangles); pan-dig2 (inverted empty triangles); FACS-dig3 (filled squares). One-tenth of the amount of shockate isolated from 20 ml OD units/cm at A_{600} (or 100 μ l) was twofold serially diluted in duplicate on digoxigenin-ovalbumin-coated ELISA wells. (No binding to ovalbumin alone was detected using these conditions.) (B) Western blot of osmotic shock and spheroplast fractions of equal amounts of cells (the same samples used for derivation of shockates used in Fig. 4A) probed with (1) anti- β -lactamase (β -lac) serum (2) anti-aminoglycoside phosphotransferase (APH) serum, and (3) anti-his⁶ serum (scFv). (1) and (2) were used as controls to demonstrate the efficiency of fractionation. Anti-rabbit-HRP (1 and 2) and anti-mouse-HRP (3) were used as secondary antibodies. (C) BIAcore analysis of 500 μ M purified monomeric pan-dig1 and FACS-dig3 scFv on a digoxin-BSA-coupled chip. Sensorgrams were subtracted from a BSA blank. (D) Monoclonal phage ELISA on digoxin-ovalbumin of culture supernatants from M13K07 infected TG-1-bearing negative control pHEN2.ctr (filled circles); pHEN2.26-10 (empty circles); pHEN2.pan-dig1 (filled inverted triangles); pHEN2.pan-dig2 (empty inverted triangles); pHEN2.FACS-dig3 (filled squares). Infected cells were grown with vigorous aeration at 25°C overnight in glucose-free 2x TY, cells pelleted, and 100 μ l of supernatant twofold serially diluted. (No binding to ovalbumin alone under these conditions was detected.)

cyanine 5-diaminopentane-trinitrobenzene (Cy5-DAP-TNB), which is positively charged and has a molecular weight of 953 Da. The best clone (TNB) exhibited a ninefold higher mean fluorescence relative to cells expressing the 26-10 scFv antibody that does not recognize the TNB hapten. Similarly, cells expressing a scFv specific for a 21-mer peptide could be labeled with the respective fluorescent peptide conjugate and affinity-matured using PECS (G. Chen, A. Hayhurst, B.R. Harvey, B.L. Iverson, and G. Georgiou, unpublished results).

To evaluate the molecular weight limit for the diffusion of ligands into the periplasmic space under the conditions identified above, we used a set of oligonucleotides bearing digoxigenin at one end and a fluorescein isothiocyanate (FITC) label at the other end. The oligonucleotides were synthesized using phosphorothioate linkages to minimize degradation by nucleases and consisted of 5 (2,384 Da), 10 (4,897 Da), or 20 (8,727 Da) adenosine residues. Upon incubation with 100 nM probe, negative control cells expressing a scFv to the unrelated hapten atrazine²⁷ showed background fluorescence distri-

bution (mean fluorescence intensity ~ 6) irrespective of the size of the probe. In contrast, cells expressing the 26-10 scFv, which binds to the digoxigenin moiety of the oligonucleotides, exhibited 5-fold, 3.5-fold, and ~ 2 -fold greater fluorescence than the control when labeled with the 5-mer, 10-mer, or 20-mer, respectively (Fig. 3B). Presumably, as the size and charge of the oligonucleotides increase, we are reaching the limits on outer membrane permeability under the conditions we used. It should be noted, however, that in our hands a twofold fluorescence difference still allows resolution and sorting of populations.

Analysis and screening of phage libraries by FACS. We have found that FACS can be used to isolate ligand-binding proteins from existing libraries constructed for display on bacteriophage. This is very useful, because the construction of highly diverse libraries *de novo* is technically demanding. Phage-displayed libraries are amenable to screening by PECS because, during phage biogenesis, pIII fusions are first targeted to the periplasm and anchored onto the inner membrane by a small C-terminal portion of pIII (refs 28,29). In the most widely used vectors for phage display, an amber codon is placed between the N-terminal scFv and the pIII gene. Thus, as a consequence of inefficient amber suppression, all libraries containing amber codons give some periplasmic expression of the proteins not fused to pIII.

Conventional phage panning of a widely used semisynthetic library (Griffin, 1) successfully enriched digoxin-binding phage (Fig. 4A, B). Pans 3 and 4 had 0/48 and 28/48 positives, respectively, as judged by monoclonal phage enzyme-linked immunosorbent assay (ELISA). Twenty-four positive clones from pan 4 shared the same DNA fingerprint. DNA sequencing of six isolates confirmed the presence of a single heavy- and light-chain family used (VH5 and VL1). One clone was represented five times ("pan-dig1"), whereas a second was found once ("pan-dig2"). Mutations responsible for differences between

pan-dig1 and pan-dig2 were in HCDR3 (P97T, S98Y), LCDR3 (R95aP, A95bR, V96-), and LFR1 (L4V).

The phage population from each round was used to infect ABLEC. Upon labeling with digoxigenin-BODIPY, the cell populations from each round displayed fluorescence distributions consistent with the corresponding phage ELISA signals (compare Fig. 4A and C). Significant enrichment of binding clones in a single round of PECS was obtained with cell populations infected with phage from the third and fourth rounds of panning. The fourth round yielded 7/8 positive for hapten binding by FACS, all clones being identical to pan-dig1. Although the third round yielded 5/14 FACS positives, 3/5 encoded a novel scFv, designated "FACS-dig3" (the remaining 2/5 were pan-dig1). This clone exhibited a completely different DNA fingerprint compared to the clones isolated by panning. Sequencing revealed a different heavy chain (VH3) and minor variations in LCDR3 (R95aG, A95bG, V96P) relative to pan-dig1. Importantly, the FACS-dig3 DNA fingerprint was never detected in two separate phage panning experiments involving the analysis of over 350 phage clones from rounds 3,

4, and 5 (those that exhibited a positive signal by conventional monoclonal phage ELISA being fingerprinted). Thus, FACS-dig3 represents a library clone that could apparently only be identified by PECS, presumably for the reasons detailed below.

The pan-dig1, pan-dig2, and FACS-dig3 scFvs were expressed in ABLE under identical conditions. Cells expressing pan-dig2 gave no FACS signal, whereas pan-dig1 and FACS-dig3 gave a comparable fluorescence signal (mean fluorescence intensities of 34 and 42, respectively). In ELISA assays of periplasmic (osmotic shock) fractions, pan-dig1 gave a signal comparable to the 26-10 scFv antibody and FACS-dig3 gave a weak signal, whereas pan-dig2 gave no signal (Fig. 5A). Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and western blotting of the same shockate samples used in the ELISA revealed that the absence of an ELISA signal by the pan-dig2 clone was due to the exclusive accumulation by the respective scFv protein in an insoluble form in the cell. The pan-dig1 scFv accumulated at moderate levels in the periplasmic fraction, whereas the clone isolated only by PECS (FACS-dig3) was expressed extremely well and was fully soluble (Fig. 5B). BIAcore analysis of purified, monomeric dig1 and dig3 scFv protein (Fig. 5C) indicated that the latter exhibits a faster hapten dissociation rate. This finding is consistent with the lower signal observed by phage ELISA with the FACS-dig3 clone (Fig. 5A). These results suggest that (1) PECS can only select clones that exhibit at least a threshold of soluble protein expression in bacteria. Such clones are, of course, most desirable for protein engineering applications. (2) Both low- and high-affinity binders can be isolated by a single PECS setup. In contrast, with phage display, higher affinity binders are isolated by monovalent (gene III) display whereas lower affinity binders generally require polyvalent (gene VIII) display. By not relying on display and adsorption for selection, PECS most likely avoids "avidity effects" that often hamper concise phage selections.

To analyze why the FACS-dig3 clone was missed in the panning experiments, PHEN2 bearing the corresponding gene was moved into TG-1 cells. TG-1 cells transformed with phagemids encoding pan-dig1, pan-dig2, FACS-dig3, 26-10 as a positive control, and the anti-atrazine scFv as a negative control, were infected with helper phage and incubated overnight at 25°C. Rescued phage were then analyzed by ELISA (Fig. 5D). FACS-dig3 gave a lower monoclonal phage ELISA signal, indicating that, when expressed as a fusion to pIII, either phage growth or hapten binding is impaired. Interestingly, a similarly lower ELISA signal was also obtained with the 26-10 scFv, even though the latter is well expressed and possesses a high hapten affinity. Thus, whereas FACS-dig3, and for that matter clones expressing the 26-10 scFv, bind to hapten and are enriched, they are ultimately missed because they give a low phage ELISA signal.

Conclusions. PECS is based on the observation that, under certain experimental conditions, fluorescently tagged ligands including synthetic small molecules, peptides, and oligonucleotides can permeate the bacterial outer membrane and become selectively concentrated when bound by a receptor protein within the periplasm. Fluorescent cells can then be isolated by flow cytometry^{1,9}.

The finding that large ligands can be made to diffuse through the bacterial outer membrane without significant loss of proteins from the periplasm is surprising, but not unprecedented. In fact, there exist physiological mechanisms whereby proteins (e.g., colicins) and even very large complex structures (e.g., filamentous phage) can cross the outer membrane in a highly selective manner. Thus, with further optimization, PECS might be used to screen for binders to larger ligands than those studied here. The system could also be applied to nucleic acid-binding proteins, potentially overcoming the toxicity of these proteins when expressed in the cytoplasm of *E. coli* by other directed evolution methods.

In this report, we have shown that PECS can be used to isolate

binders with subnanomolar dissociation constants. Importantly, existing phage display libraries can be screened by PECS. Clones that would have been missed by standard panning and screening approaches, particularly very well-expressed clones, are readily isolated in this manner. By definition, PECS selects only for clones that exhibit good expression characteristics in the host. We are currently using PECS for the isolation of scFvs to small molecules other than digoxin, for the affinity maturation of anti-peptide antibodies, and, finally, for expression maturation of toxic or aggregation-prone proteins (A. Hayhurst, B.R. Harvey, B.L. Iverson, and G. Georgiou, unpublished results). Finally, it should be noted that the work described here was performed using a low-throughput benchtop flow cytometer. With a high-throughput flow cytometer (e.g., a Cytomation MoFlo) or perhaps by employing highly parallel microfluidic sorters³¹, it will be possible to screen very large libraries including mammalian complementary DNA libraries and naïve antibody repertoires. The screening of such libraries can be performed using repeated rounds of PECS without intermediate amplifications of the sorted cells, thus affording significant timesaving over phage panning.

Experimental protocol

Fluorescent probes and conjugates. The synthesis of digoxigenin-BODIPY has been described^{32,33}. The fluorescent conjugate of TNBS was a derivative of Cy5 (Amersham Pharmacia, Piscataway, NJ). Conjugates of TNB and protein were made by combining 100-fold molar excess of TNBS with ovalbumin or BSA, allowing the mixture to react at room temperature for 2 h, and then dialyzing extensively against PBS. Phosphorothioate linkage oligonucleotides bearing a 5'-digoxigenin ester and a 3'-FITC were from Integrated DNA Technologies (Coralville, IA) and supplied at reverse-phase high-performance liquid chromatography (RP-HPLC) purity.

Strains and plasmids. *Escherichia coli* strains TG1 and HB2151 were provided by the Griffin library. *Escherichia coli* ABLE was purchased from Stratagene (La Jolla, CA), and helper phage M13K07 was purchased from Amersham Pharmacia. *Escherichia coli* LMG194 (*ara*) and plasmid pBAD30 were gifts from Jon Beckwith. Plasmid pET25b was from Novagen (Madison, WI). Information on the construction of expression vectors and restriction maps of the plasmids used in this work can be provided by the authors upon request.

Affinity maturation of the A4-19 scFv. A library of 10⁶ transformants in which LCDR3 residues Thr91, Val94, and Pro96 in the A4-19 anti-digoxin scFv had been randomized using synthetic oligonucleotides³⁴ was created. The cells were grown at 37°C overnight and were then subcultured in fresh Luria-Bertani (LB) medium and grown at 25°C for 2 h. The cells were induced with 0.2% arabinose for 4 h and then were incubated with digoxigenin-BODIPY (100 nM) for 1 h. After washing once in PBS buffer, labeled cells were sorted on a Becton Dickinson FACSort (San Jose, CA). The cells were first sorted in recovery mode, collected in PBS and subsequently poured into SOC medium for overnight growth at 37°C. The diluted cells were grown at 25°C and induced with arabinose for 4 h. The cells were labeled with 100 nM probe, then incubated with 5 µM of unlabeled digoxin for between 15 and 90 min and sorted in exclusion mode. Again, the cells were grown in SOC overnight at 37°C and individual clones subsequently selected from plating dilutions of the saturated culture.

Surface plasmon resonance. The association constants of scFv mutants were measured according to the method of Chen *et al.*⁹ using a BIAcore 1000 instrument.

PECS Optimization. An aliquot of ABLE bearing the clone of interest was scraped from a glycerol stock into 1 ml of 2x TY (2% glucose, 100 µg/ml ampicillin) to obtain an OD₆₀₀ of ~0.1. After 2 h of growth at 37°C with vigorous shaking, scFv expression was induced with 1 mM isopropyl-β-D-galactoside (IPTG), and the culture was transferred to 25°C for 4 h. Fifty microliters of culture were labeled with 100 nM of fluorescent probe in 1-ml of 1x PBS (10 mM Na₂HPO₄, 1.8 mM KH₂PO₄, 2.7 mM KCl, 137 mM NaCl), 2.5x PBS, 5x PBS, or 10x PBS for 1 h at room temperature with moderate agitation. For the last 10 min of labeling, P1 was added to 1 µg/ml. Cells were pelleted, resus-



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pendent in 100 µl of the same buffer, and the fluorescence distribution of 10⁴ events were analyzed at ~1500 s⁻¹ on the FACS Sort. The analysis of TNB-Cys conjugate was done on a MoFlo (Cytomation, Fort Collins, CO).

Phage panning and FACS sorting of phage libraries. The Griffin.1 library was rescued and subjected to five rounds of panning essentially according to the website instruction manual (<http://www.mrc-cpe.cam.ac.uk/phage/glp.html>). Immunotubes were coated with 10 µg/ml conjugate overnight at 4°C in 50 mM carbonate buffer pH 9.3. Following each pan, the neutralized triethylamine eluates were halved and used to infect either TG-1 for the next round or ABLEC for FACS analysis. Eluate titers were monitored to indicate enrichment of antigen-binding phage.

For phage library sorting, the cells were grown in terrific broth, induced with 0.1 mM IPTG, and labeled with 100 nM probe in 5x PBS. Sorting was performed in exclusion mode on 10⁶ events (10⁷ for round 2) at no more than 1,000 events/s. The collected sort solution was passed through 0.2 µm membrane filters (Millipore Corporation, Bedford, MA), and cells withheld by the filters were allowed to grow after placement on SOC agar plus appropriate antibiotics at 30°C for 24 h.

Analysis of PECS-isolated clones from phage libraries. Small-scale fractionation and western blotting was performed according to Hayhurst and Harris²⁷. A 10 µl aliquot from a 1 ml fraction derived from 20 ml OD units/cm at A₆₀₀ was electrophoresed on a 12% Laemmli gel using the Bio-Rad (Hercules, CA) mini-protein cell. Samples were electroblotted to Immobilon P (Millipore) and probed with rabbit anti-β-lactamase or anti-APH-II sera (5 prime-3 prime, Boulder, CO) followed by anti-rabbit horseradish peroxidase (HRP) conjugate (Bio-Rad). ScFv proteins were detected using mouse anti-His6 (Sigma, St. Louis, MO) followed by anti-mouse HRP (Bio-Rad). Detection was carried out using SuperSignal West Pico chemiluminescent substrate (Pierce, Rockford, IL). ELISA was done on 100 µl of periplasmic fraction, or a

100 µl aliquot of phage supernatant using nonfat dried milk as a blocking agent and anti-his6/anti-mouse HRP for detecting scFv and anti-M13-HRP (Amersham Pharmacia) for detecting phage. ELISA points represent the average of duplicate wells. The entire fractionation and ELISA process was carried out three times on separate occasions and, while the absolute values varied slightly, the trend was always as shown herein.

Periplasmic expression and purification of scFvs. We used 40 ml terrific broth cultures of clones of interest to produce scFv for biophysical characterization. Arabinose or lac promoter-based vectors were induced with either 0.2% L-arabinose or 0.1 mM IPTG, respectively. Expression was allowed to continue for 4 h at 25°C. Protein was purified from osmotic shock fluid using immobilized metal affinity chromatography followed by size exclusion chromatography (Superdex-75, Pharmacia) to isolate monomeric scFv. Protein concentration was determined by A₂₈₀ using an extinction coefficient of 44,850 or bicinchoninic acid assay using BSA as a standard.

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